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(71) Applicant: NITTO CHEMICAL INDUSTRY CO., LTD.
Tokyo (JP)

(72) Inventors:

- Yu, Fuji
Yokohama-shi, Kanagawa 227 (JP)
- Kato, Mami
Yokohama-shi, Kanagawa 235 (JP)

(74) Representative: VOSSIUS & PARTNER

Siebertstrasse 4
D-81675 München (DE)

Remarks:

The applicant has subsequently filed a sequence listing and declared, that it includes no new matter.

(54) **A kanamycin resistance gene derived from microorganisms of the genus rhodococcus**

(57) The present invention relates to a DNA derived from microorganisms of the genus Rhodococcus and conferring kanamycin resistance on hosts with a DNA sequence coding for the amino acid sequence of Sequence No. 1 or a polypeptide containing a partial sequence thereof. The kanamycin resistance gene of the present invention is useful to construct vectors for microorganisms of the genus Rhodococcus, particularly vectors for self-cloning of Rhodococcus rhodochrous.

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Description

The present invention relates to a gene derived from microorganisms of the genus *Rhodococcus* and conferring kanamycin resistance on bacteria, as well as a plasmid vector containing the same.

Microorganisms belonging to the genus *Rhodococcus* are known as bacterial catalysts that hydrate or hydrolyze nitriles to the corresponding amides or acids (Japanese Patent Publication No. 4873/92 and Japanese Laid-Open Patent Publication Nos. 91189/87, 470/90 and 84198/90), and in particular, microorganisms belonging to the species *Rhodococcus rhodochrous* possess nitrile-hydrating activity of extremely high performance (Japanese Laid-Open Patent Publication No. 470/90).

Under such circumstances, one of the present inventors found cryptic plasmids in a certain strain of the species *Rhodococcus rhodochrous* and constructed hybrid plasmid vectors to develop a host-vector system of the genus *Rhodococcus* (Japanese Laid-Open Patent Publication Nos. 148685/92, 64589/93 and 68566/93).

For construction of a self-cloning system of higher safety, it is also necessary to develop marker genes derived from microorganisms of the genus *Rhodococcus*. However, only arsenious acid and cadmium resistance genes derived from microorganisms of the species *Rhodococcus rhodochrous* are known as such drug resistance genes (Plasmid 23, 242-247 (1990)).

With the aim of establishing a self-cloning system of the genus *Rhodococcus*, the present inventors extensively studied drug resistance genes derived from microorganisms of the genus *Rhodococcus*, in particular the species *Rhodococcus rhodochrous*, so that they found the kanamycin resistance gene of the present invention.

That is, the present invention relates to a gene derived from microorganisms of the genus *Rhodococcus* and conferring kanamycin resistance on hosts, wherein said gene codes for the amino acid sequence of Sequence No. 1 or a polypeptide containing a partial sequence thereof.

The present invention furthermore relates to a gene conferring kanamycin resistance on a host comprising the DNA sequence of Sequence No. 2 or a DNA sequence which

- (a) differs from said DNA sequence due to the degeneracy of the genetic code;
- (b) hybridizes with said DNA sequence or the DNA sequence of (a); or
- (c) represents a fragment, allelic or other variation of the above DNA sequence, whether said variation results in changes in the polypeptide sequence or not.

In this context, the term "hybridization" refers to conventional hybridization conditions, preferably to stringent hybridization conditions.

FIG. 1 shows a restriction enzyme map of plasmid pKM001.

FIG. 2 shows the construction of plasmid pKM002, pKM003 and pKM004.

FIG. 3 shows a restriction enzyme map of plasmid pKM011.

As the DNA donor in the present invention, mention may be made of kanamycin mutant KM-02 (deposited as FERM BP-5137 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan) which was obtained by spontaneous mutation of *Rhodococcus rhodochrous* ATCC 12674.

As the vectors used in cloning in the present invention, mention may be made of plasmid vectors including but not limited to *E. coli* vectors such as pTrc99A, pUC18, etc. and phage vectors such as λ gt11 etc. The host microorganisms include but are not limited to *E. coli* JM109, *E. coli* JM105, and *Rhodococcus rhodochrous* ATCC 12674.

Plasmids that provide plasmid vectors constructed of the kanamycin resistance gene of the invention with a region capable of replicating in microorganisms of the genus *Rhodococcus* include, but are not limited to, plasmids pRC001, pRC002, pRC003 and pRC004. The plasmids pRC001, pRC002, pRC003 and pRC004 are derived from *Rhodococcus rhodochrous* ATCC 4276, ATCC 14349, ATCC 14348 and IFO 3338, respectively, and these plasmids are described in the aforementioned Japanese Laid-Open Patent Publication Nos. 148685/92, 64589/93 and 68566/93, respectively.

The present kanamycin resistance gene derived from microorganisms of the genus *Rhodococcus* is useful to construct vectors for microorganisms of the genus *Rhodococcus*, particularly vectors for self-cloning of *Rhodococcus rhodochrous*.

The present invention is described in more detail with reference to the following examples, which however are not intended to limit the scope of the present invention.

Example 1

Cloning of Kanamycin Resistance Gene from Mutant KM-02 into *E. coli* JM109

5 (1) Preparation of genomic DNA from KM-02 and preparation of a DNA library

The KM-02 strain was cultured under shaking at 30 °C in 100 ml MY medium (0.5 % polypeptone, 0.3 % Bacto-yeast extract, 0.3 % Bacto-malt extract) and genomic DNA was prepared from the bacteria according to the method by Saito and Miura (Biochim. Biophys. Acta 72, 619 (1963)). A part of the resulting DNA was partially digested with restriction
10 enzyme Sau3AI and then inserted into a BamHI site of *E. coli* vector pTrc99A to give a recombinant DNA library.

(2) Preparation of transformants and selection of recombinant DNA

The recombinant library prepared in step (1) was used to transform *E. coli* JM109 by the calcium chloride method,
15 and transformants with resistance to kanamycin were selected in the following manner.

The transformants obtained above were plated onto LB agar medium (1 % Bacto-trypton, 0.5 % Bacto-yeast extract, 0.5 % NaCl, 1.5 % agar) containing 40 µg/ml kanamycin hydrochloride and 1 mM IPTG (isopropyl-β -thiogalactoside) and incubated overnight at 37 °C. The colonies occurring thereon were removed and applied onto the same agar medium, and their growth was confirmed.

20 A plasmid DNA was prepared from the thus obtained transformant according to the method by Birnboim and Doly (Nucleic Acid Res. 7, 1513-1523 (1979)) and designated pKM001. This plasmid was reintroduced into *E. coli*, and the resultant transformant with kanamycin resistance was designated JM109/pKM001 and deposited as FERM BP-5138 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology. IPTG was required for expression of Kanamycin resistance of *E. coli* JM109/pKM001.

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(3) A restriction enzyme map of pKM001 and location of the kanamycin resistance gene

A restriction enzyme map of plasmid pKM001 obtained in step (2) was prepared (FIG. 1). Thereafter, this plasmid pKM001 was used for preparing plasmids of a smaller DNA fragment. The target gene-containing region was identified
30 by the presence or absence of the kanamycin resistance of transformants prepared in the same manner as in step (2). During this process, plasmid pKM002 (FIG. 2) was constructed.

(4) Nucleotide sequencing

35 The nucleotide sequence of the kanamycin resistance gene in plasmid pKM002 was determined by Fluorescence Sequencer ALF II produced by Pharmacia (Sequence No. 3).

Example 2

40 Preparation of Hybrid (*E. Coli*-*Rhodococcus*) Plasmid Vector Carrying the Kanamycin Resistance Gene Derived from *Rhodococcus Rhodochrous*

A hybrid plasmid vector pK4, previously constructed by one of the present inventors by ligating *Rhodococcus*-derived plasmid pRC004 with *E. coli* vector pHSG299 and deposited as FERM BP-3731 with the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology (Japanese Laid-Open Patent Publication Nos. 64589/93 and 68566/93), was used for preparing a 3.1 kb HindIII fragment containing the whole of pRC004 and a part of pHSG299, and the resulting fragment was ligated with the plasmid pKM002.

45 As a result, two plasmids carrying the insert in the opposite direction were obtained and designated pKM003 and pKM004, respectively (FIG. 2). These plasmids replicate in both the genus *Rhodococcus* and *E. coli*. *Rhodococcus rhodochrous* ATCC 12674 was transformed with these plasmids by electroporation, whereby a transformant capable of growing in MY medium containing 75 µg/ml kanamycin was obtained. The plasmids obtained from the transformant were the same plasmids as those introduced. Where microorganisms of the genus *Rhodococcus* were used as the host, the presence of IPTG was not required for expression of kanamycin resistance.

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Example 3

Construction of Vector for Microorganisms of the Genus Rhodococcus

5 The hybrid plasmid vector pKM004 was cleaved with restriction enzyme KpnI to give a 4.3 kb KpnI fragment which was then self-ligated and introduced into Rhodococcus rhodochrous ATCC 12674 by electroporation. The resulting transformant showed the same degree of kanamycin resistance as did the transformant of Example 2. From this trans-

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formant, a plasmid was obtained and designated pKM011 (FIG. 3).

SEQUENCE TABLE

5 Sequence No: 1

 Length: 171

 Sequence Type: amino acid

10 Topology: linear

 Nature: protein

 Origin

15 Microorganism: Rhodococcus rhodochrous

 Strain: KM-02

20 Sequence:

Met Ser Asp Asn Gly Ser Gly Thr Thr Arg Pro Glu Gly Ala Pro Leu

 1 5 10 15

25 Pro Arg Arg Ala Arg Ser Ser Arg Pro Ser Ala Gly Asn Ser Pro Ala

 20 25 30

30 Pro Gly Arg Arg Ala Val Val Ala Lys Ser Arg Arg Arg Leu Ala Ala

 35 40 45

 Ala Pro Glu Ala Gly Thr Thr His Tyr Ser Ile Phe His Gly Asp Gln

35 50 55 60

 Leu Ile Gly Phe Ile Gln Trp Tyr Glu Ala Glu Asp Asn Pro Asp Phe

 65 70 75 80

40 Arg His Ala Gly Leu Asp Leu Phe Leu Asp Pro Asp Phe His Gly Arg

 85 90 95

 Gly Phe Gly Arg Glu Ser Ile Arg Val Leu Cys Ala His Leu Ile Asp

45 100 105 110

 Asp Leu Ala Phe His Arg Leu Val Ile Asp Pro Glu Val Asp Asn Ser

50 115 120 125

 Val Ala Ile Ala Cys Tyr Arg Ser Val Gly Phe Lys Asp Val Gly Val

 130 135 140

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Sequence No: 2

Length: 516

Nature: nucleic acid

Strand Form: double-stranded

Topology: linear

Origin

Microorganism: Rhodococcus rhodochrous

Strain: KM-02

Sequence:

ATG AGT GAC AAC GGC TCC GGA ACT ACG CGG CCC GAG GGT GCT CCT CTC	48
CCC CGT CGC GCC CGA TCA TCA CGC CCG TCT GCG GGC AAT TCA CCT GCA	96
CCC GGA CGT CGT GCA CTG GTG GCA AAA TCC CGA CGA CGA CTG GCT GCG	144
GCG CCA GAA GCC GGA ACC ACG CAC TAC AGC ATC TTC CAC GGC GAC CAA	192
CTG ATC GGC TTC ATC CAG TGG TAC GAA GCG GAA GAC AAC CCC GAC TTC	240
GCG CAC GCC GGG CTC GAC TTG TTC CTC GAT CCC GAC TTC CAC GGC CGA	288
GGG TTC GGT CGC GAA TCG ATT CGC GTG CTG TGT GCC CAC CTG ATC GAC	336
GAC CTC GCA TTC CAC CGT CTG GTC ATC GAC CCG GAG GTC GAC AAC TCC	384
GTC GCC ATC CCG TGC TAC CGA TCG GTG GGG TTC AAA GAC GTC GGG GTG	432
ATG CGC GAG TAT TCG CGA GAT CGC CAT GGT GTG TGG AAG GAC GGA CTG	480
CTG ATG GAT CTG CTC GCA CGG GAA TTC ATC CGC TGA	516

Sequence No: 3

Length: 748

Sequence Type: nucleic Acid

Strand Form: double-stranded

Topology: linear

Origin

Microorganism: Rhodococcus rhodochrous

Strain: KM-02

5 Sequence:

GGATCCGGGG TCGTCCGCCA CCAGGATGGT ACCCAAGCCG GGTGTGATGC CCTCTGCCCTT 60

10 CGAGCGCTCA CCCGCACCTT CAGGTCTTCC AAGATTTCGT CGCGGGTAGC TTTGCCGTCC 120

AGGATCGTTG CAGTCACGGC GACCATTGTT CCAGGTTAGG GTCGATGAGT GACAACGGCT 180

CCGGAACCTAC GCGGCCCCGAG GGTGCTCCTC TCCCCCGTCG CGCCCGATCA TCACGCCCGT 240

15 CTGCGGGCAA TTCACCTGCA CCCGGACGTC GTGCAGTGGT GGCAAAATCC CGACGACGAC 300

TGGCTGCGGC GCCAGAAGCC GGAACCACGC ACTACAGCAT CTTCCACGGC GACCAACTGA 360

20 TCGGCTTCAT CCAGTGGTAC GAAGCGGAAG ACAACCCCGA CTTCCGCCAC GCGGGGCTCG 420

ACTTGTTCCT CGATCCCGAC TTCCACGGCC GAGGGTTCCG TCGCGAATCG ATTCCCGTCC 480

TGTGTGCCCC CCTGATCGAC GACCTCGCAT TCCACCGTCT GGTGATCGAC CCGGAGGTCC 540

25 ACAACTCCGT CGCCATCGCG TGCTACCGAT CGGTGGGGTT CAAAGACGTC GGGGTGATGC 600

GCGAGTATTC GCGAGATCGC CATGGTGTGT GGAAGGACGG ACTGCTGATG GATCTGCTCG 660

30 CACGGGAATT CATCCGCTGA TCGACTGGGA CGAGTTCGAA AGGACCGACA TCATGTTGCT 720

GGACAAGGAA TTCACGGCCA CCCTGCAG 748

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Nitto Chemical Industry Co., Ltd.
- (B) STREET: 5-1, Marunouchi 1-chome, Chiyoda-ku
- (C) CITY: Tokyo
- (E) COUNTRY: Japan
- (F) POSTAL CODE (ZIP): 100

(ii) TITLE OF INVENTION: A kanamycin resistance gene derived from microorganisms of the genus rhodococcus

(iii) NUMBER OF SEQUENCES: 3

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: EP 95 11 2298.5

(vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: JP 201582/1994
- (B) FILING DATE: 04-AUG-1994

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 171 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Rhodococcus rhodochrous
- (B) STRAIN: KM-02

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Met Ser Asp Asn Gly Ser Gly Thr Thr Arg Pro Glu Gly Ala Pro Leu
1 5 10 15

Pro Arg Arg Ala Arg Ser Ser Arg Pro Ser Ala Gly Asn Ser Pro Ala
20 25 30

Pro Gly Arg Arg Ala Val Val Ala Lys Ser Arg Arg Arg Leu Ala Ala

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	35	40	45
5	Ala Pro Glu Ala Gly Thr Thr His Tyr Ser Ile Phe His Gly Asp Gln		
	50	55	60
	Leu Ile Gly Phe Ile Gln Trp Tyr Glu Ala Glu Asp Asn Pro Asp Phe		
	65	70	75 80
10	Arg His Ala Gly Leu Asp Leu Phe Leu Asp Pro Asp Phe His Gly Arg		
	85	90	95
	Gly Phe Gly Arg Glu Ser Ile Arg Val Leu Cys Ala His Leu Ile Asp		
	100	105	110
15	Asp Leu Ala Phe His Arg Leu Val Ile Asp Pro Glu Val Asp Asn Ser		
	115	120	125
	Val Ala Ile Ala Cys Tyr Arg Ser Val Gly Phe Lys Asp Val Gly Val		
20	130	135	140
	Met Arg Glu Tyr Ser Arg Asp Arg His Gly Val Trp Lys Asp Gly Leu		
	145	150	155 160
25	Leu Met Asp Leu Leu Ala Arg Glu Phe Ile Arg		
	165	170	

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 516 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: linear
- (vi) ORIGINAL SOURCE:
- (A) ORGANISM: Rhodococcus rhodochrous
 - (B) STRAIN: KM-02

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

	ATGAGTGACA ACGGCTCCGG AACTACGCGG CCCGAGGGTG CTCCTCTCCC CCGTCGCGCC	60
45	CGATCATCAC GCCCGTCTGC GGGCAATTCA CCTGCACCCG GACGTCGTGC AGTGGTGGCA	120
	AAATCCCGAC GACGACTGGC TCGGCGCCA GAAGCCGGAA CCACGCACTA CAGCATCTTC	180
50	CACGGCGACC AACTGATCGG CTTTCATCCAG TGGTACGAAG CGGAAGACAA CCCCAGCTTC	240
	CGCCACGCCG GGCTCGACTT GTTCCTCGAT CCCGACTTCC ACGGCCGAGG GTTCGGTCGC	300
55	GAATCGATTG GCGTGCTGTG TGCCACCTG ATCGACGACC TCGCATTCCA CCGTCTGGTC	360

ATCGACCCGG AGGTGACAA CTCCGTCGCC ATCGCGTGCT ACCGATCGGT GGGGTTCAAA 420
 5 GACGTCGGGG TGATGCGCGA GTATTGCGCA GATCGCCATG GTGTGTGGAA GGACGGACTG 480
 CTGATGGATC TGCTCGCACG GGAATTCATC CGCTGA 516

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 748 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: double
 (D) TOPOLOGY: linear

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Rhodococcus rhodochrous*
 (B) STRAIN: KM-02

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GGATCCGGGG TCGTCGCCCA CCAGGATGGT ACCCAAGCCG GGTGTGATGC CCTCTGCCTT 60
 CGAGCGCTCA CCCGCACCTT CAGGTCTTCG AAGATTTCGT CGCGGGTAGC TTTGCCGTCG 120
 30 AGGATCGTTG CAGTCACGGC GACCATTGTT CCAGGTTAGG GTCGATGAGT GACAACGGCT 180
 CCGGAACTAC GCGGCCCCGAG GGTGCTCCTC TCCCCCGTCG CGCCCGATCA TCACGCCCCG 240
 CTGCGGGCAA TTCACCTGCA CCCGGACGTC GTGCAGTGGT GGCAAAATCC CGACGACGAC 300
 35 TGGCTGCGGC GCCAGAAGCC GGAACCACGC ACTACAGCAT CTTCCACGGC GACCAACTGA 360
 TCGGCTTCAT CCAGTGGTAC GAAGCGGAAG ACAACCCCGA CTTCCGCCAC GCCGGGCTCG 420
 40 ACTTGTTTCCT CGATCCCGAC TTCCACGGCC GAGGGTTCGG TCGCGAATCG ATTCGCGTGC 480
 TGTGTGCCCA CCTGATCGAC GACCTCGCAT TCCACCGTCT GGTGATCGAC CCGGAGGTCG 540
 ACAACTCCGT CGCCATCGCG TGCTACCGAT CGGTGGGGTT CAAAGACGTC GGGGTGATGC 600
 45 GCGAGTATTC GCGAGATCGC CATGGTGTGT GGAAGGACGG ACTGCTGATG GATCTGCTCG 660
 CACGGGAATT CATCCGCTGA TCGACTGGGA CGAGTTCGAA AGGACCGACA TCATGTTGCT 720
 50 GGACAAGGAA TTCACGGCCA CCCTGCAG 748

55 Claims

1. A gene derived from a microorganism of the genus *Rhodococcus* and conferring kanamycin resistance on a host, said gene coding for the amino acid sequence of Sequence No. 1 or a polypeptide containing a partial sequence thereof.

2. The gene according to claim 1, comprising the DNA sequence of Sequence No. 2 or a partial sequence thereof.

3. A gene conferring kanamycin resistance on a host and comprising a DNA sequence which

- 5 (a) differs from the DNA sequence of claim 2 in the codon sequence due to the degeneracy of the genetic code;
(b) hybridizes with the DNA sequence of claim 2 or section (a), above; or
(c) represents a fragment, allelic or other variation of the DNA sequence of claim 2, whether said variation results in changes in the polypeptide sequence or not.

10 4. The gene according to any one of claims 1 to 3, wherein the host microorganism is a microorganism of the genus Rhodococcus or Escherichia coli.

5. A plasmid vector comprising a gene according to any one of claims 1 to 4 and a DNA region capable of replicating in a microorganism of the genus Rhodococcus.

15 6. The plasmid vector according to claim 5, wherein the DNA region capable of replicating in a microorganism of the genus Rhodococcus is derived from a plasmid selected from pRC001, pRC002, pRC003 or pRC004.

7. A host cell transformed with the plasmid of claim 5 or 6.

20 8. The host cell of claim 7, which is a cell of a microorganism of the genus Rhodococcus or Escherichia coli.

9. Use of the gene of any one of claims 1 to 4 as a marker for the construction of a self-cloning system.

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FIG. 1

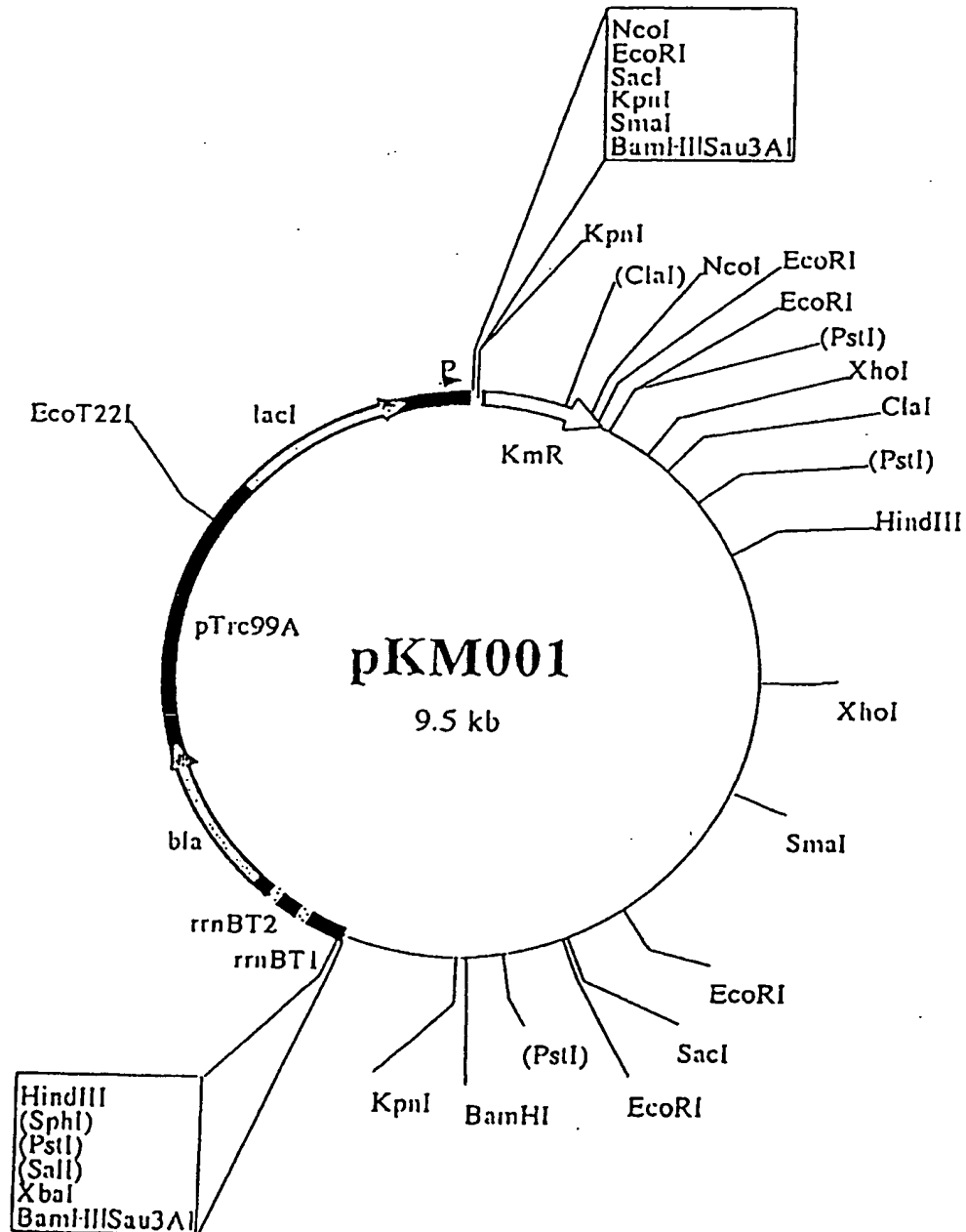


FIG. 2-b

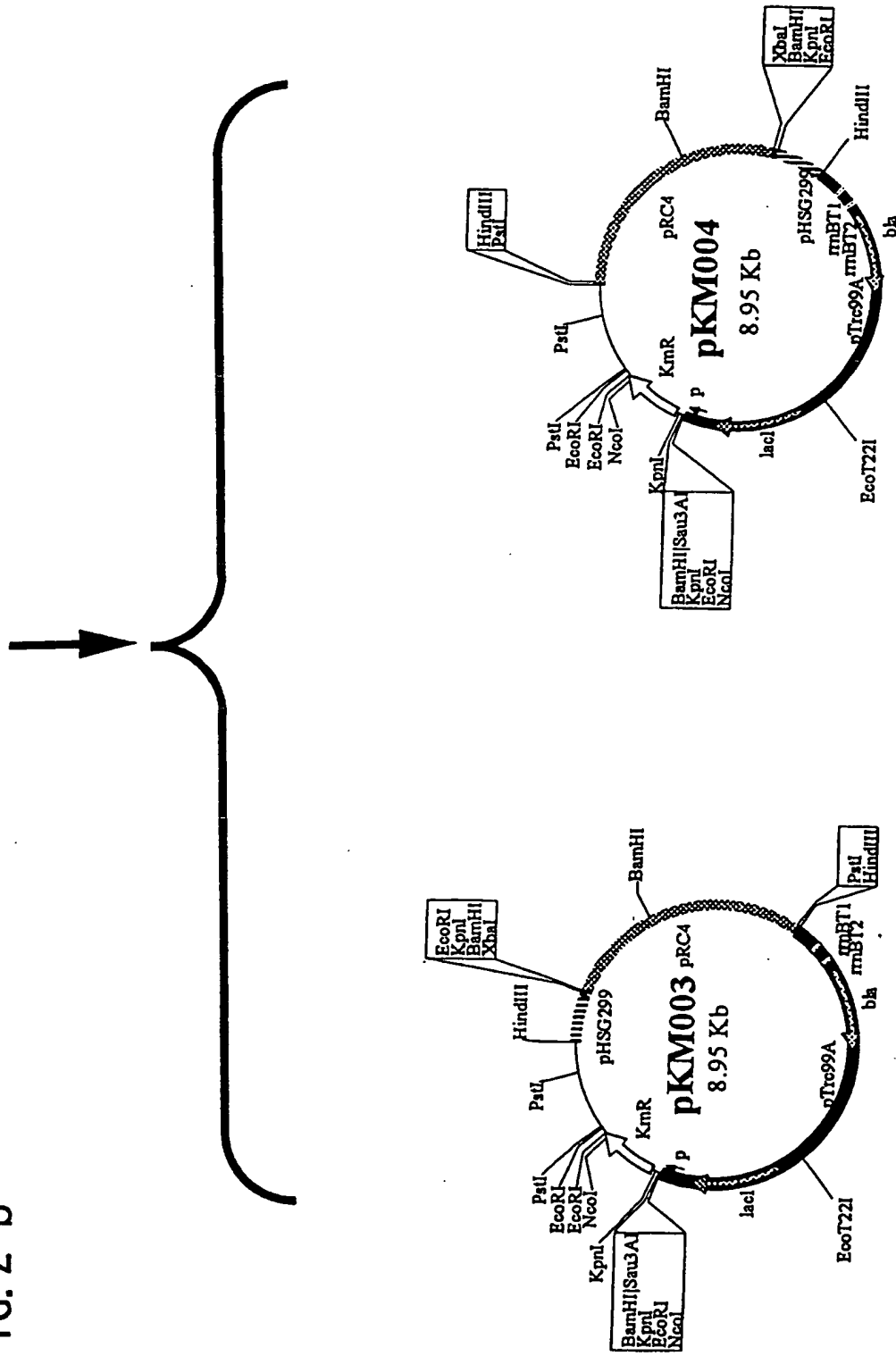


FIG. 3

